JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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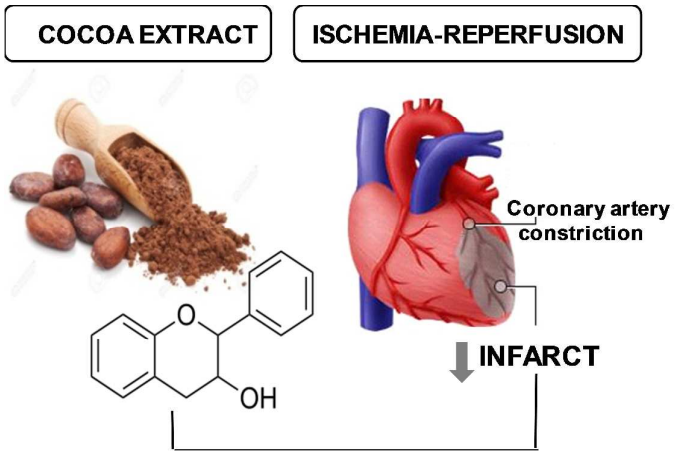
J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b01669 • Publication Date (Web): 09 Jun 2016

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

1 **TOCgraphicabstract**



EX VIVO TREATMENT WITH A POLYPHENOL-ENRICHED COCOA EXTRACT AMELIORATES MYOCARDIAL INFARCT AND POSTISCHEMIC MITOCHONDRIAL INJURY IN NORMOTENSIVE AND HYPERTENSIVE RATS.

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Conflict of Interest

The authors declare that they have no conflict of interest.

ABSTRACT

Our objective was to determine the effects of a polyphenol-enriched cocoa extract (PCE) on myocardial postischemic alterations in normotensive (Wistar rats, W) and spontaneously hypertensive rats (SHR). Isolated hearts were submitted to 110 min of perfusion or 20-min stabilization, 30-min global ischemia and 60-min reperfusion (R). Other hearts were treated with PCE at the onset of R. Infarct size, the reduced glutathione (GSH) and the expression of phospho-Akt, P-GSK-3β and P-eNOS were assessed. In isolated mitochondria the Ca^{2+}- mediated response of mitochondrial permeability transition pore (mPTP), membrane potential ($\Delta\psi m$) and superoxide production were determined. PCE decreased infarct size, partly preserved GSH, increased the P-Akt, P-GSK-3β and P-eNOS contents, improved mPTP response to Ca^{2+}, decreased the superoxide production and restored $\Delta\psi m$.

These data show that PCE decreases the cardiac postischemic damage in W rats and SHR and suggest that Akt/GSK-3β/eNOS dependent pathways are involved.

Key words: Wistar, SHR, infarct size, mitochondria, polyphenols

INTRODUCTION

Cocoa and chocolate are two products derived from processing of cocoa beans. This complex multistage process begins with spontaneous fermentation driven in the postharvest period by different microorganisms derived from the environment [1]. After fermentation cocoa beans are roasted, shelled, and ground. The main difference between cocoa and chocolate is the absence or existence of cocoa butter. In cocoa, butter is little or non-existent. In contrast, chocolate has butter. Therefore, cocoa is considered as a healthy drink because it has less sugar and fat and besides possesses an important amount of flavanols [2], being catechins and epicatechins the main [3, 4]. There are many evidences regarding the beneficial actions of chocolate and cocoa on immune functions, ageing, blood pressure regulation, atherosclerosis, insulin resistance, physical performance or cardiovascular diseases development. However, the molecular mechanisms remain under investigation and the subject of ongoing discussion [5-9].

The ischemic heart disease is an important cause of death worldwide [10], being the high blood pressure an important risk factor. Although the reperfusion reduces the mortality, it introduces an additional injury. Thus, many studies demonstrate that drugs or strategies applied at the beginning of reperfusion are able to reduce infarct size [11-14]. It has also been previously showed that hypertrophy consequent to chronically elevated blood pressure aggravates the reperfusion injury [15, 16].

Mitochondrial integrity is critical in the maintenance of bioenergetics and Ca^{2+} homeostasis of the myocardium. Upon reperfusion the mitochondrial Ca^{2+} overload leads to myocyte death by multiple mechanisms including oxidative injury and opening of the mitochondrial permeability transition pore (mPTP) [10]. Therefore, the inhibition of mPTP at the beginning of reperfusion may prevent cell death and thus reduce infarct size [17-19].

Epidemiological evidences indicate that the consumption of flavonoids-rich foods or beverages decreases the incidence of cardiovascular disease [20-22]. Different studies showed the benefits of cocoa on the prevention of CVD, the ability to modulate the blood pressure in hypertensive animals and the capacity to improve coronary circulation in healthy adults [23-27]. Recently, Cienfuegos-Jovellanos et al. [28] developed a cocoa powder with the highest flavonoid monomer content. The antihypertensive effect exerted by that cocoa powder (PCE) has been previously demonstrated by the same authors [29]. However, its action during ischemia-reperfusion is still unknown.

75 The purpose of this study was to examine the actions of ¨ex vivo¨ treatment with PCE, administered at the
76 beginning of reperfusion, on infarct size and mitochondrial state in hearts from normotensive and
77 spontaneously hypertensive rats submitted to ischemia-reperfusion.
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MATERIAL AND METHODS

Animals

Male SHR and W rats were used. The animals were housed 4 per cage, with food and drinking water ¨ad libitum¨. The room ventilation rate was 4-6 changes per h, at temperature of 22 ± 2 °C and with a light cycle/dark of 12 h. All procedures followed during this investigation were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, University of La Plata (P-05-2014).

Systolic blood pressure measurement

Systolic blood pressure (SBP) was measured in alive and awake animals by a modified tail-cuff method [30].

Polyphenol-Enriched Cocoa Extract (PCE)

The preparation, characteristics and composition of PCE are described in a previous paper [31]. Briefly, PCE was obtained from CocoanOX [32] and produced from unfermented, blanch-treated, and roasted cocoa beans. By HPLC, the total polyphenol content was 547 ± 4 mg/dry matter being the 50% represented by the total flavan-3-ol content followed by (-)-epicatechin (26%) and procyanidin B2 (15%).

Isolated heart preparation

Rats were anesthethized with ketamine-diazepam (80-5 mg/Kg). Arreflexia appearance with loss of corneal reflex and the flexor reflex of escape in the lower limbs were verified before heart isolation. Isolated hearts were perfused following the instructions previously detailed [13].

Experimental protocols

After 20-min stabilization, the following experimental protocols were performed: *Non-ischemic control hearts* (NIC; n = 5 for each rat strain): hearts were perfused for 90 min without any treatment. *Ischemic control hearts* (IC, n = 7 for each rat strain): hearts were subjected to 30 min of global ischemia followed by 60 min of reperfusion. *PCE* (n = 7 for each rat strain): hearts were treated during 10 min at the onset of reperfusion with PCE (30 µg/mL). Other hearts (n = 4 for each rat strain and for each protocol) were used for biochemical determinations and others (n = 4 for each protocol and for each rat strain) for mitochondria isolation.

Infarct size determination

Infarct size was assessed by the triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Munich, Germany) staining technique. At the end of reperfusion hearts were frozen, cut into six transverse slices and incubated in TTC. Infarct size was expressed as a percentage of total area (area at risk) [33].

Lipid peroxidation

A portion of left ventricle (LV) was homogenized and centrifuged at 3000 rpm. In the supernatant, the concentration of thiobarbituric acid reactive substances (TBARS) was measured and expressed in nmol/mg of protein [34].

Reduced glutathione (GSH)

GSH content was determined in the supernatant using the Ellman´s reagent [35] and expressed as μg/mg of protein.

Immunoblotting

Other portion of LV was homogenized and cytosolic fraction was isolated by differential centrifugation. Briefly, supernatant proteins were resolved on SDS-PAGE, transferred to PVDF membrane, blocked and probed with antibodies against phosphorylated forms of GSK-3β-Ser9, Akt, eNOS-Ser1177, anti-MnSOD and anti-Cytochrome c. Protein bands were analyzed by a chemiluminescent system and total GSK-3β, Akt and eNOS content or GAPDH signal were used as a loading control [13].

Isolation of mitochondria

LV of other sets of control and treated hearts from W rats and SHR were used to mitochondria isolation following the method previously described [13].

Ca^{2+}- induced mPTP opening

The isolated mitochondria were energized and induced to swell with the addition of $CaCl_2$. If mPTP opens the mitochondria swells. These changes are observed as decreases of light scattering (LSD) at 520 nm using a temperature-controlled Hitachi F4500 spectrofluorometer [36]. LSD was assessed in samples without any treatment and in those treated with PCE 10 μg/ml.

Mitochondrial membrane potential

Mitochondrial potential (ΔΨm) was evaluated by measuring rhodamine-123 (RH-123) fluorescence quenching [37] and calculated following the instructions previously detailed [38].

Measurements of $O_2^{\cdot-}$ production

Superoxide production was measured in intact mitochondria suspension with lucigenin-enhanced chemiluminiscence (CL) as previously described [39]. The CL in arbitrary units (a.u.) was recorded with a

luminometer (Chameleon, Hidex, Tuku, Finland) for 10 sec each one with 1 min interval during 10 min in the presence or absence of succinate (6 mM/L) or PCE (10 µg/ml). Mitochondrial $O_2^{\cdot-}$ production was expressed as a.u./min/mg protein.

Statistical analysis

Data were expressed as means ± SE. Differences between groups were assessed with a two-way analysis of variance (ANOVA) test and Newman-Keul's was used as a post hoc test. A value of $p < 0.05$ was considered to be statistically significant.

148 **RESULTS**

149 Mean data of systolic blood pressure (SBP) plus the values of body weight (BW, g), left ventricular
150 weight (LVW, mg) and hypertrophic index (HI, calculated as LVW and BW ratio) of W rats and SHR are
151 displayed in Table 1. SBP, LVW and HI were significantly higher in SHR than W rats, indicating the
152 presence of hypertrophy associated to high pressure as one recognized characteristic of hypertensive
153 animals.

154 ***Infarct size***

155 Hearts from W rats and SHR without any treatment caused an infarct size of ~30% of the risk area. When
156 PCE was added to the perfusate a significant reduction in infarct size was obtained (Fig. 1).

157 ***TBARS and GSH***

158 The TBARS concentration- as an index of lipid peroxidation- of IC hearts was 0.75 ± 0.06 and 0.97 ±
159 0.10 nmol/mg protein for W rats and SHR, respectively. These values were not significantly modified by
160 PCE treatment (0.61 ± 0.08 and 0.70 ± 0.15 nmol/mg protein for W rats and SHR, respectively). The
161 GSH content in non-ischemic control hearts from SHR was lower than that detected in hearts from W
162 rats. After ischemia-reperfusion, GSH levels decreased to a similar value in hearts from SHR and W rats.
163 The treatment with PCE partially or fully preserved the GSH content in hearts from normotensive and
164 hypertensive rats, respectively (Fig. 2).

165 ***Expression of P-Akt, P-GSK-3β and P-eNOS***

166 At the end of reperfusion period, homogenates of PCE treated hearts from W rats and SHR showed a
167 significant increase of the expression of phosphorylated forms of Akt, e-NOS and GSK-3β (Fig. 3).

168 ***MnSOD and cytochrome c***

169 The loss of internal mitochondrial membrane impermeability leads to the release of mitochondrial matrix
170 components, as MnSOD and cytochrome c, to cytosol. Thus, the expression of both substances increased
171 in ischemic control hearts from W rats and SHR and decreased in PCE treated hearts from both rats
172 strains (Fig. 4).

173 ***Ca^{2+}- induced mPTP opening (LSD) and mitochondrial membrane potential ($\Delta\Psi m$)***

174 Figure 5 shows the typical traces (A panel) and mean values (B panel) of light scattering decrease (LSD)
175 produced by the addition of 100 μmol/L Ca^{2+} to mitochondrial suspensions of untreated and treated hearts
176 from W rats and SHR. LSD was significantly lesser in non-ischemic hearts from SHR in comparison to
177 those of W rats. After ischemia-reperfusion, the LSD decreased to a similar value for hearts from both

178 rats strains. The treatment with PCE improved the response of mitochondria to Ca^{2+} showing greater LSD
179 values than ischemic hearts but lesser than those observed in non-ischemic hearts. Figure 6 shows the
180 changes of $\Delta\psi m$ in the three experimental protocols. The $\Delta\psi m$ of mitochondria isolated from SHR hearts
181 was significantly lesser than those of W rats. After ischemia-reperfusion the $\Delta\psi m$ decreased in both rats
182 strains. The treatment with PCE attenuated this depolarization reaching $\Delta\psi m$ values not statistically
183 different to those obtained in non-ischemic control hearts but maintaining the difference between W rats
184 and SHR.

185 ***Mitochondrial $O_2^{\cdot-}$ production***

186 As shown in Figure 7 the incubation of cardiac mitochondria with lucigenin elicited a basal $O_2^{\cdot-}$
187 production in W and SHR. This response appears to be due to the presence of endogenous substrates in
188 the freshly isolated mitochondria. The addition of succinate significantly enhanced the $O_2^{\cdot-}$ production in
189 mitochondria from both rats strains and decreased after treatment with PCE.
190

DISCUSSION

The present data showed that the ¨ex vivo¨ treatment at the onset of reperfusion with a polyphenol-enriched cocoa extract (PCE) decreased the cell death and attenuated the mitochondrial injury produced by ischemia-reperfusion in hearts from normotensive and spontaneously hypertensive rats.

Hypertension is an important cause of cardiovascular morbidity and mortality and it has been associated with impaired antioxidant defense and specially with disturbances in glutathione metabolism [40, 41]. This was evident in our study since we found lesser GSH values in non-ischemic control hearts from SHR in comparison to W rats. The treatment with PCE partially or fully preserved the level of GSH in hearts from W rats and SHR, respectively. Additionally, a decreased $O_2^{\cdot-}$ production in isolated mitochondria from PCE-treated hearts of both rats strains was showed. These results suggest that a reduced ROS production and/or higher scavenging could be taking place in cardiac tissue from normotensive and hypertensive animals when they were submitted to ischemia and reperfusion in presence of PCE.

On the other hand, and in agreement with a recent paper published by us [42], the $\Delta\Psi m$ of mitochondria isolated from SHR hearts was less electronegative than that detected in hearts from W rats. After ischemia and reperfusion, the mitochondria suffered depolarization, reaching a similar $\Delta\Psi m$ in both rats strains. The treatment with PCE normalized $\Delta\Psi m$, maintaining the difference between W rats and SHR.

The mitochondrial permeability transition pore (mPTP) plays a critical role in determination of cell death and is the focal point of the various protective mechanisms [43-45]. The mPTP opening leads to matrix swelling and efflux of cyc and other proapoptotic factors [46, 47]. Our data show that the Ca^{2+}- mediated response of mitochondria isolated from non-ischemic control hearts of W rats was higher than those of SHR, diminished to a similar value when hearts were submitted to ischemia-reperfusion and was partially restored in PCE treated hearts. Therefore, the restoration of $\Delta\Psi m$ and the Ca^{2+} response are indicators of an improvement of mitochondrial state mediated by PCE. In our conditions, we also detected an increase of MnSOD and cyc expression in ischemic control hearts from W rats and SHR which decreased after PCE treatment. All these data are evidence of the protective role of cocoa extract against mitochondria permeability and suggest that an attenuation of ROS production and a diminution of Ca^{2+} uptake by mitochondria could be the responsible mechanisms.

A relevant piece of information is how processes occurring in the cytosol modulate mPTP opening. Which are the PCE targets?. GSK-3β phosphorylation is a step to which multiple protective signaling pathways converge ending to avoid the mPTP opening [48]. In our experimental conditions, the treatment

with PCE increased the level of phospho-GSK-3β in both rats strains suggesting that the PCE-mediated cardioprotection is linked to GSK-3β-dependent mechanism. Among the kinases able to activate GSK-3β is the PI3K/Akt which has been involved in the beneficial actions during ischemia-reperfusion [49]. We also observed a decrease of phospho- Akt level whereas opposite changes took place in PCE treated hearts.

Several papers have demonstrated the protective role of NO during ischemia-reperfusion [50, 51]. It is recognized that the balance of NO concentration depends of its production by increase of eNOS expression and/or activity and the $O_2^{-\cdot}$ formation in which the eNOS uncoupling plays an important role [52]. In our experimental conditions, PCE increased the expression of phosphoSer1177-eNOS, which linked to the reduced $O_2^{-\cdot}$ production could lead to a higher NO bioavailability in PCE-treated compared to untreated hearts. Therefore, the data present herein show, by the first time, that NO could be an important mediator of the infarct size limitation afforded by PCE.

PCE contains four times more procyanidins and eight times more epicatechin and procyanidin B2 than conventional cocoa powder [31]. There is accumulating evidence that (−)-epicatechin and its derivatives have significant role in prevention of CVD in humans [53]. Potent antioxidant action, modulation of cell signalling, reduction of the blood pressure, and protection of mitochondria, are being proposed as possible mechanisms of beneficial effects of (−)-epicatechin [54]. The ability of (−)-epicatechin to prevent oxidative stress by restoring NO bioavailability was has been also showed [55]. Recently, it was demonstrated that (−)-epicatechin and procyanidin B2 improve mitochondrial functions detecting a decrease of cyc release [56]. As these compounds are present in high proportion in PCE, it might be responsible for the beneficial effects detected in PCE-treated hearts.

In summary, our findings show that the ¨ex vivo¨ treatment of PCE at the onset of reperfusion ameliorates the infarct size in hearts from W rats and SHR by attenuation of mPTP opening and suggest that Akt/eNOS and Akt/GSK-3β-dependent signaling pathways are involved. Thus, our data are providing arguments to establish the benefits of PCE against the mitochondrial impairment produced by ischemia-reperfusion. A decrease of ROS production by mitochondria plus to the scavenging activity of the extract which leads to the preservation of GSH levels could be contributing to the cardioprotective action (Fig. 8).

Limitations

In the current study we demonstrated, by the first time, in a model of heart ¨ex vivo¨ the beneficial action of a polyphenol-enriched cocoa extract against reperfusion injury. However, the complex composition of

the extract and the low intestinal absorption of its constituents determine that our findings could not be extrapolled directly to human. Furthermore, long-term trials will be needed to investigate the incidence of PCE addition to diet on clinical outcomes of patients suffering adverse cardiovascular events.

ABBREVIATIONS

Cyc: Cytochrome c

eNOS: Endothelial nitric oxide synthase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GSK-3β: Glycogen synthase kinase-3 beta

IC: Ischemic control hearts

MnSOD: Manganese-dependent superoxide dismutase

mPTP: Mitochondrial permeability transition pore

ΔΨm: Mitochondrial potential

NIC: Non-ischemic hearts

W: Normotensive Wistar rats

PCE: Polyphenol-enriched cocoa extract

ROS: Radical oxygen species

GSH: Reduced glutathione

Akt: Serine/threonine-specific protein kinase

SHR: Spontaneously hypertensive rats

$O_2^{\cdot-}$: Superoxide anion

TBARS: Thiobarbituric acid reactive substances

TTC: Triphenyltetrazolium chloride

FUNDING SOURCES

This study was supported by the Grant M-169 from the National University of La Plata of Argentina to Dr. Susana Mosca.

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430

Legends

Figure 1: A panel: Scheme of ischemic control (IC) and polyphenol-enriched cocoa extract (PCE) protocols and representative slices of hearts from normotensive (W) and spontaneously hypertensive rats (SHR) stained with TTC. B panel: Mean values of infarct size (IS), expressed as a percentage of risk area, in IC (n = 7) and PCE (n = 7) treated hearts from W rats (n = 14) and SHR (n = 14). Observe that the treatment with PCE decreased the IS detected in IC hearts of both rats strains. * $p < 0.05$ vs. IC

Figure 2: Reduced glutathione content (GSH, µg/mg protein) in non-ischemic control (NIC, n = 4), ischemic control (IC, n = 4) and PCE (n = 4) treated hearts from normotensive (W, n = 12) and spontaneously hypertensive rats (SHR, n = 12). The GSH content diminished in IC and it was partially or fully preserved in W rats and SHR, respectively, in PCE treated hearts . ϕ $p < 0.05$ SHR vs. W; * $p< 0.05$ vs. NIC; # $p < 0.05$ vs. IC.

Figure 3: Representative immunoblots of total and phosphorylated forms and summary of densitometry data of phospho-Akt (A panel), phospho-eNOS (B panel) and phospho- GSK-3β (C panel) in non-ischemic control (NIC, n = 4), ischemic control (IC, n = 4) and PCE (n = 4) treated hearts from W rats (n = 12) and SHR (n = 12). The P-Akt/Akt, P-eNOS/eNOS and P-GSK-3β/ GSK-3β ratios diminished in IC and increased in PCE treated hearts of both rats strains. * $p< 0.05$ vs. NIC; # $p< 0.05$ vs. IC.

Figure 4: Expression of MnSOD (A panel) and cytochrome c (cyc, B panel) in non-ischemic control (NIC, n = 4), ischemic control (IC, n = 4) and PCE (n = 4) treated hearts from W rats (n = 12) and SHR (n = 12). Note that a significant increase of MnSOD and cyc was detected in IC hearts from both rats strains which returned to basal values by PCE treatment. * $p < 0.05$ vs. NIC; # $p < 0.05$ vs. IC.

Figure 5: A panel: Typical traces produced by 100 µM Ca^{2+} addition to samples of mitochondria from W rats and SHR hearts. B panel: Mean values of the light scattering decreases (LSD) after Ca^{2+} addition, expressed in arbitrary units (a.u.), in non-ischemic control (NIC, n = 4), ischemic control (IC, n = 4), and PCE (n = 4) treated hearts from W rats (n = 12) and SHR (n = 12). The response of isolated mitochondria to Ca^{2+} significantly diminished in IC hearts and partially recovered after PCE treatment in both rats strains. ϕ $p < 0.05$ SHR vs. W; * $p < 0.05$ vs. NIC; # $p < 0.05$ vs. IC.

Figure 6: Mitochondrial membrane potential (ΔΨm, mV) measured in isolated mitochondria from normotensive (W, n = 12) and spontaneously hypertensive rats (SHR, n = 12) hearts of non-ischemic control (NIC, n = 4), ischemic control (IC, n = 4) and PCE treated group (n = 4). The depolarization

460 detected after ischemia and reperfusion was attenuated in PCE treated hearts. ϕ $p < 0.05$ SHR vs. W; * p
461 < 0.05 vs. NIC; # $p < 0.05$ vs. IC.

462 **Figure 7:** A and C panels: Time course of $O_2^{\cdot-}$ production of cardiac mitochondria isolated from W rats
463 and SHR, in presence or absence (C, n = 3 for W and n= 3 for SHR) of succinate (S, n = 3 for each rat
464 strain) or S + PCE (n = 3 for each rat strain). The chemiluminiscence response was initiated by adding of
465 lucigenin. B and D panels: Mean values of $O_2^{\cdot-}$ production at 3 min in C, S and S + PCE mitochondrial
466 suspensions of W rats and SHR. PCE decreased the $O_2^{\cdot-}$ production in both rats strains. * $p < 0.05$ vs. C;
467 # $p < 0.05$ vs. PCE.

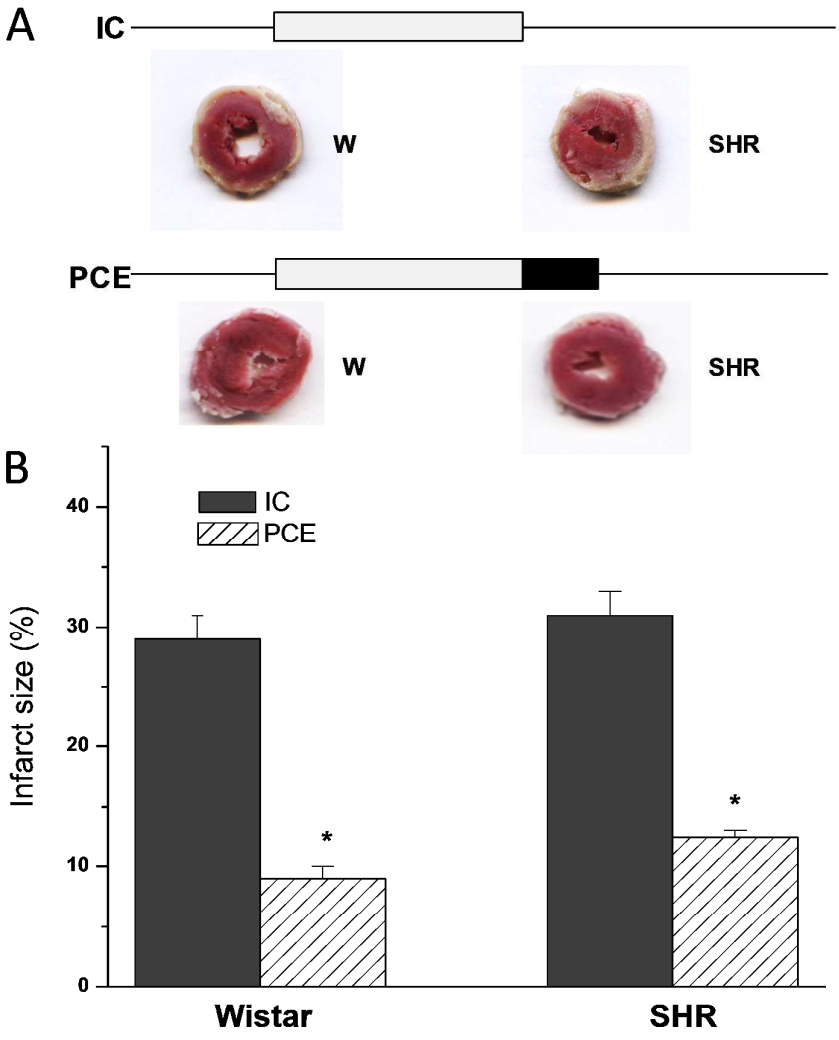
468 **Figure 8:** Scheme showing the signaling pathways that involve activation of kinases and enzyme leading
469 to the polyphenol-enriched cocoa extract (PCE)-mediated cardioprotection highlighting the mitochondrial
470 effects.

Table 1: Data of systolic blood pressure (SBP), body weight (BW), left ventricular weight (LVW) and hypertrophy index (IH) in W and SHR

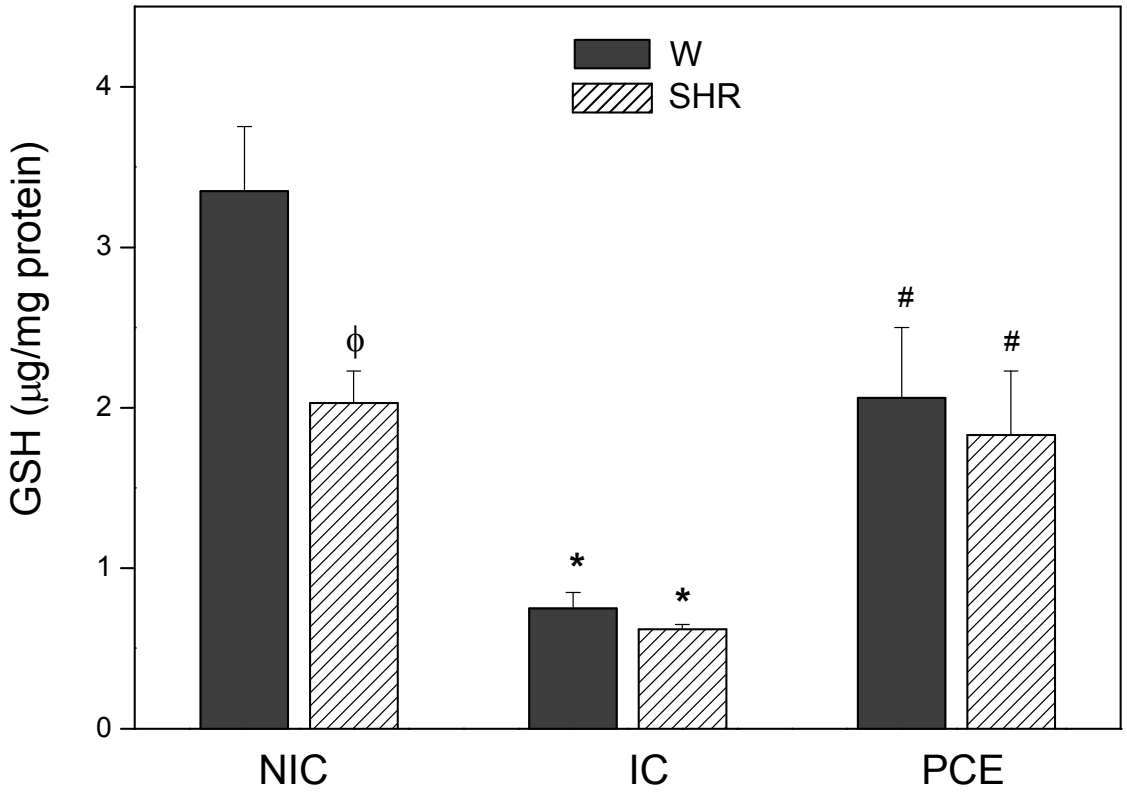
	W	**SHR**
SBP (mmHg)	125 ± 2	219 ± 3**
BW (g)	309 ± 9	310 ± 8
LVW (mg)	780 ± 40	1330 ± 60**
HI	2.52 ± 0.12	4.17 ±0.18**

**$p < 0.01$ n = 30 for each one

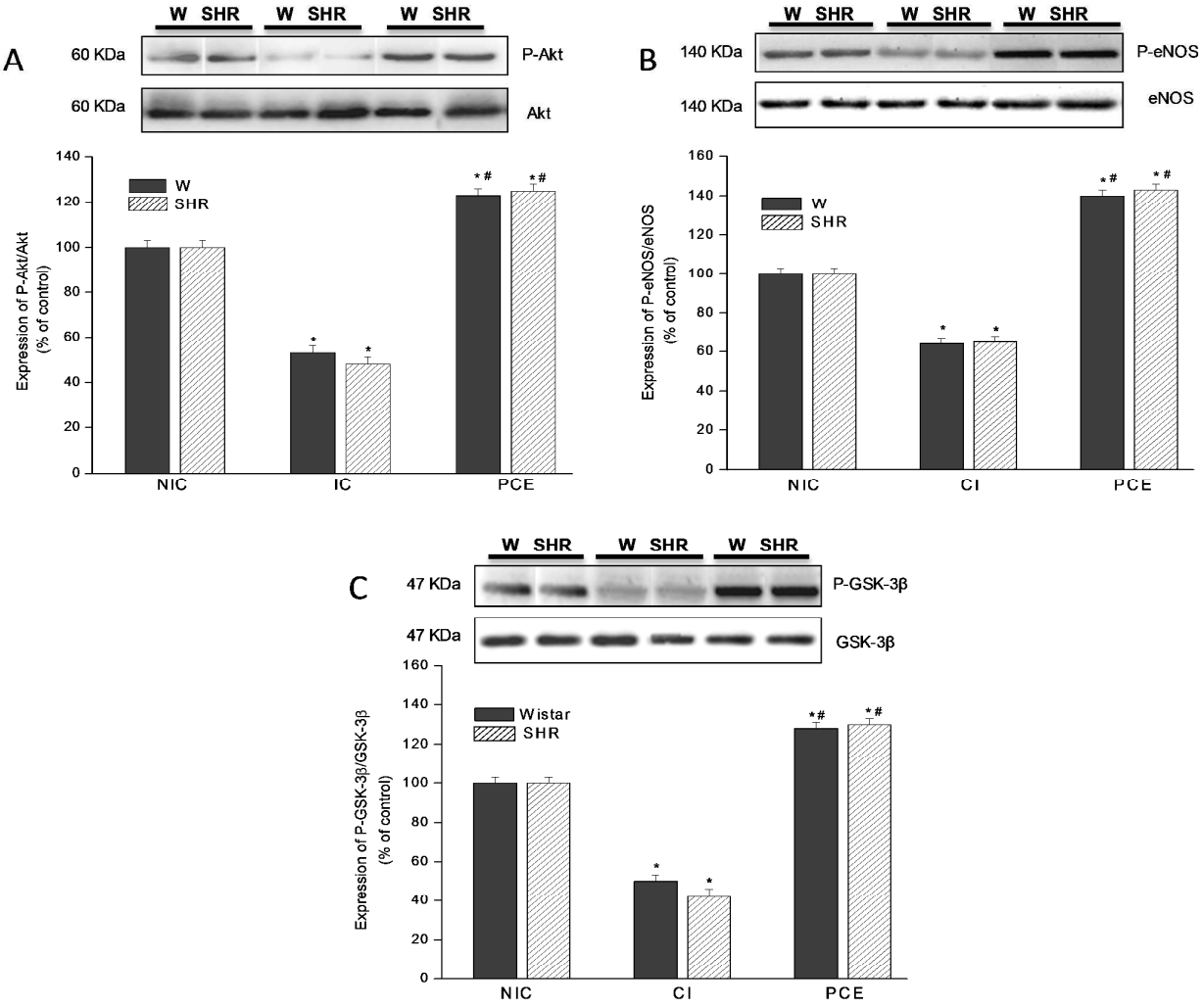
480 FIGURE 1
481



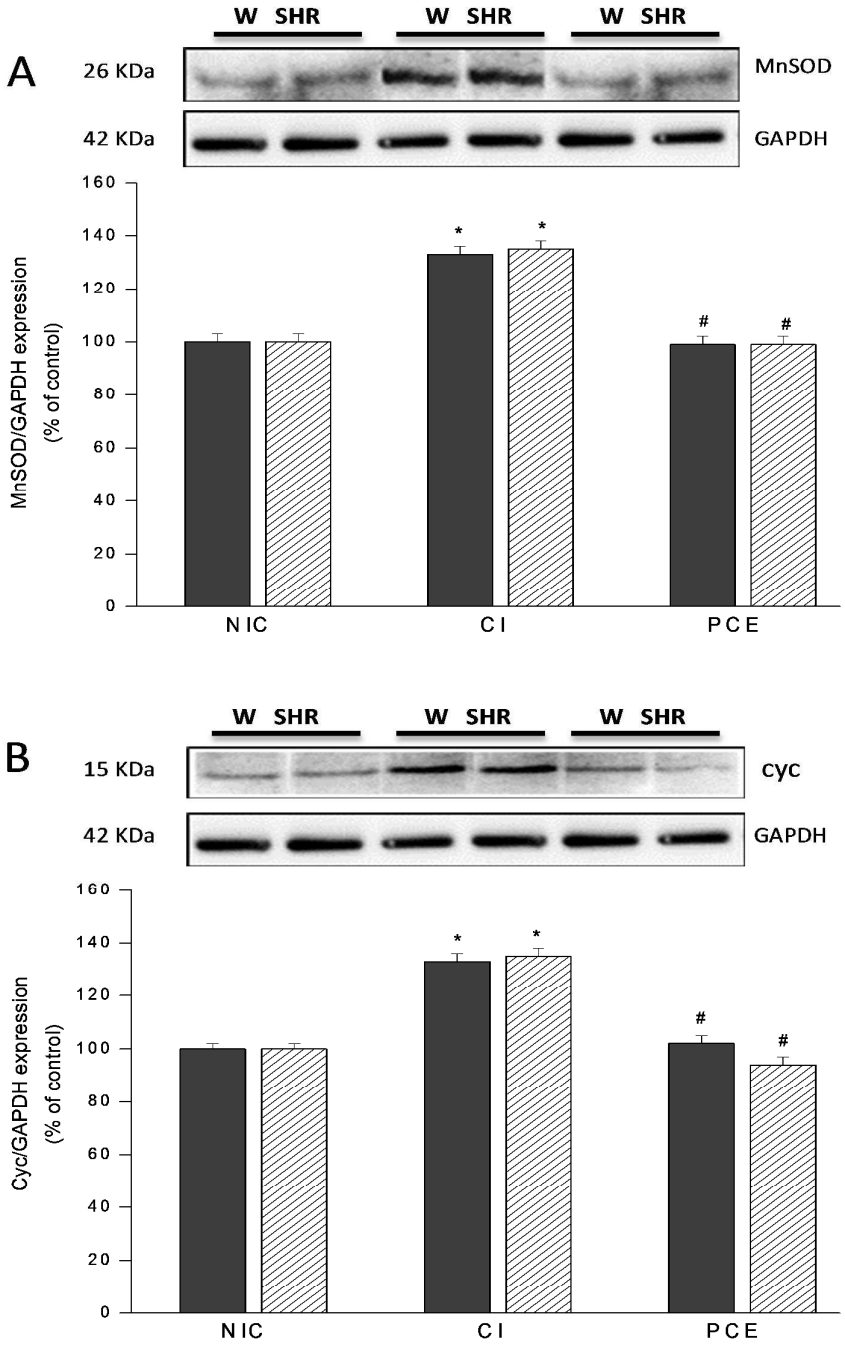
482 FIGURE 2
483



484 FIGURE 3


A
W SHR W SHR W SHR
60 KDa
P-Akt
60 KDa
Akt
Expression of P-Akt/Akt (% of control)
W
SHR
NIC
IC
PCE
B
W SHR W SHR W SHR
140 KDa
P-eNOS
140 KDa
eNOS
Expression of P-eNOS/eNOS (% of control)
W
SHR
NIC
CI
PCE
C
W SHR W SHR W SHR
47 KDa
P-GSK-3β
47 KDa
GSK-3β
Expression of P-GSK-3β/GSK-3β (% of control)
Wistar
SHR
NIC
CI
PCE

485 FIGURE 4
486



487 FIGURE 5
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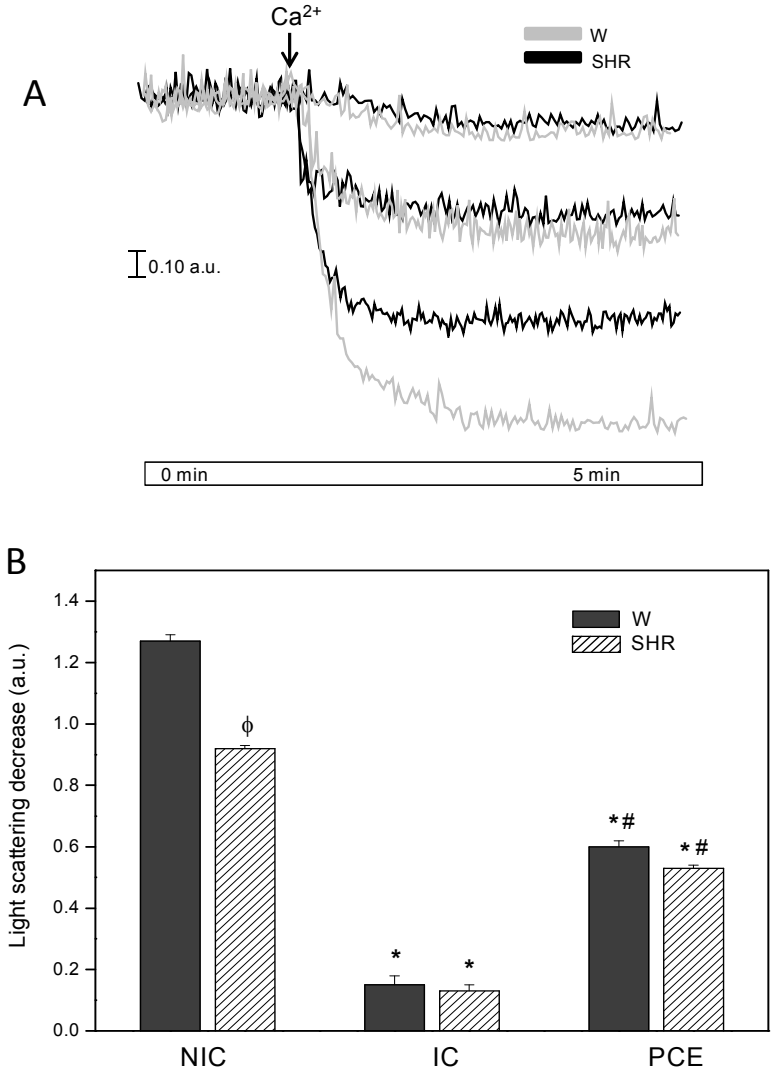

489 FIGURE 6
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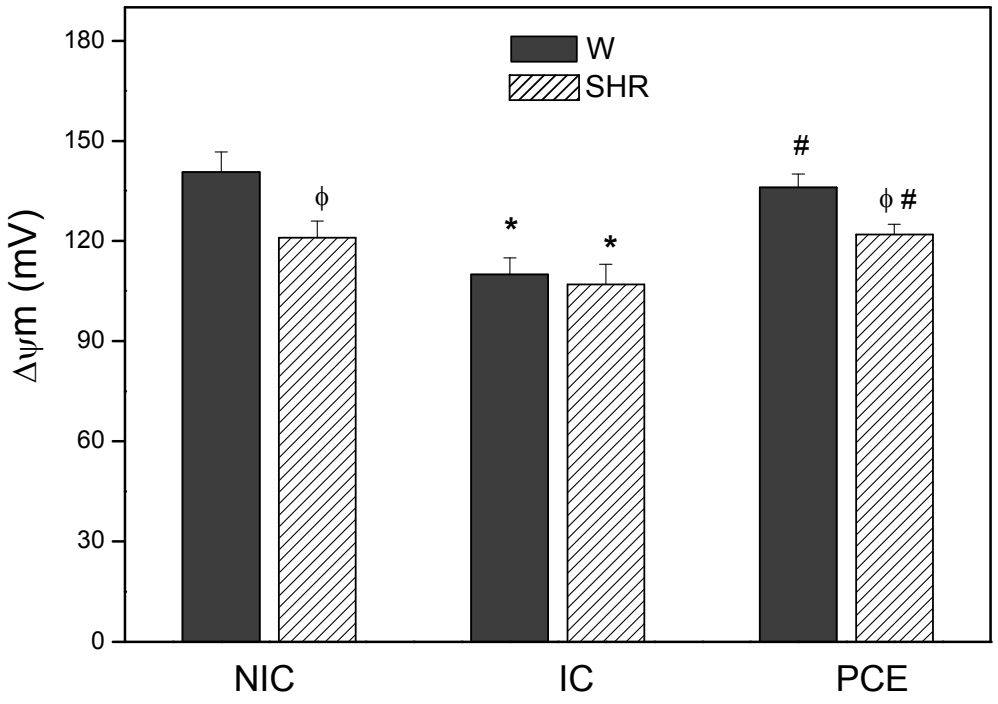

491 FIGURE 7
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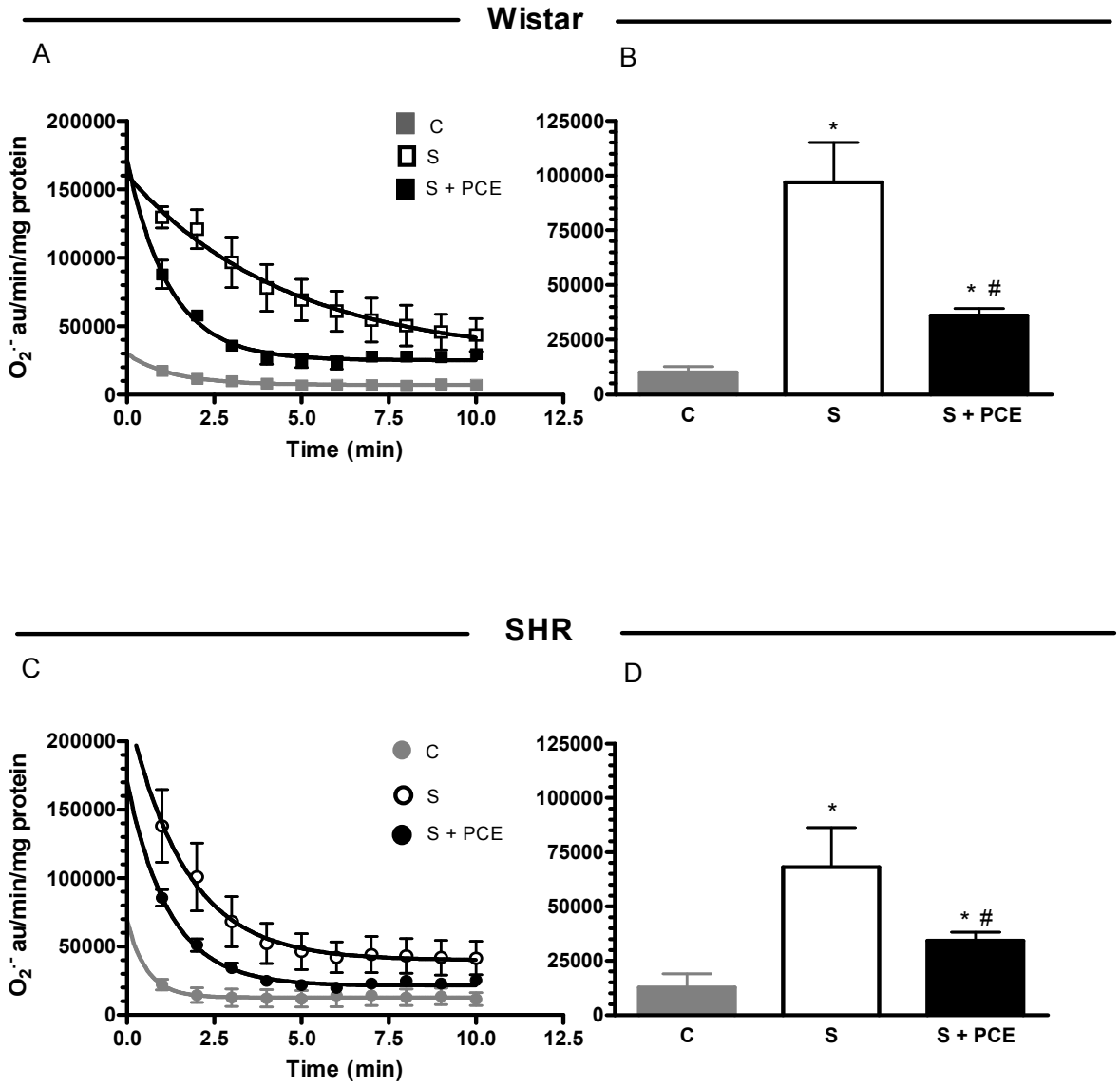

493 FIGURE 8
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